A Rapid and Sensitive Method for Identifying Permeable Areas in the Body Wall of Insects

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In 1954 the writer found that the tips of the long, thick-walled sensory pegs on the antennae and other parts of the grasshopper were permeable to acid fuchsin in aqueous solution when this was applied to the external surface of the living insect. The method as first described was altered in later papers (Slifer, 1955a, 1955b) but several difficulties remained. The dye was sometimes lost during dehydration of the part being studied either to the external solutions or to the fluids inside the sense organs. The faint pink color of small amounts of the dye was poorly visible against the natural yellow or pale brown of the unstained cuticle. Certain sense organs which, as other evidence indicated, were almost certainly provided with permeable regions, could not be shown to stain. The method described here avoids these difficulties. No dehydrating agents are used and the only clearing agent used is xylol. Rapid diffusion of the dye is prevented by using fixed material and the purple color of the dye selected is one rarely, if ever, encountered in insect cuticle.

The specimens to be examined should be clean and uninjured. Although insects caught outdoors may be used the presence of dust, pollen and other debris on their surface will make study more difficult, especially for the beginner, since these foreign materials will also stain. If broken hairs or other damaged spots are present dye will enter through these. Another source of trouble is food regurgitated by the insect during handling or fixation. This sometimes adheres to the outer surface of the body and attempts to remove it later may break hairs or abrade the cuticle. Insects which have completed the final stages of their development in the laboratory and have been isolated in clean containers are most satisfactory although even here dried residue from the molting fluid may cause confusion since it, too, will stain. However, after the investigator has gained experi-

ence by studying clean, undamaged specimens the interpretation of material which is less perfect presents few hazards.

The insect to be studied should be anesthetized very lightly with CO_2, KCN or some other agent which will not affect cuticular waxes. As soon as the insect is quiet it is dropped whole into Bouin's solution or a 10% solution of formalin and allowed to remain there for 24 hours or longer. The anaesthetic prevents the insect from struggling and damaging itself when placed in the fixative. After fixation is complete the specimen is washed well and placed entire, if it is a small one, in a 0.5% solution of crystal violet. If the insect is large the desired part—antenna, leg, head, etc.—may be removed and placed between layers of glass cloth or cotton gauze in such a way that the cut end protrudes from between the layers. Dye is then added cautiously until the cloth is saturated with it but none is permitted to reach the cut surface of the part being stained. Such preparations should be kept in a moist chamber for if water is lost crystals of dye may form on the surface of the specimen and cause trouble later.

The optimum time for staining must be determined for each new species studied. Five minutes in the dye is sufficient for some of the more delicate forms while two hours, or even longer, may be required for others. If several individuals of the same species are placed in the stain together one may be removed after five minutes and the others after 15, 30, 60 and 120 minutes—or earlier if the first specimens show good staining. If left too long in the stain all of the internal tissues as well as the inner layers of the cuticle will be colored deep purple and no information will be gained concerning sites of penetration.

Upon removal from the dye the specimen is dipped very quickly into two changes of distilled water and laid at once on a piece of absorbent paper on the stage of the dissecting microscope. The insect is moved immediately to a dry spot on the paper and the antennae, legs, mouthparts or other region of special interest cut off and moved away to dry as quickly as possible. The use of a 100 or 150 watt electric lamp to illuminate the stage is convenient for it provides heat to hasten drying.

The pieces are watched under the microscope and a few moments after all surface moisture has disappeared each specimen is transferred to a dish of xylol and left there until it clears. If the part is small it will clear within an hour or two and may then be mounted permanently on a slide in resin. If clearing proceeds very slowly the piece may be removed, drained quickly on absorbent paper and returned to xylol. While in xylol the

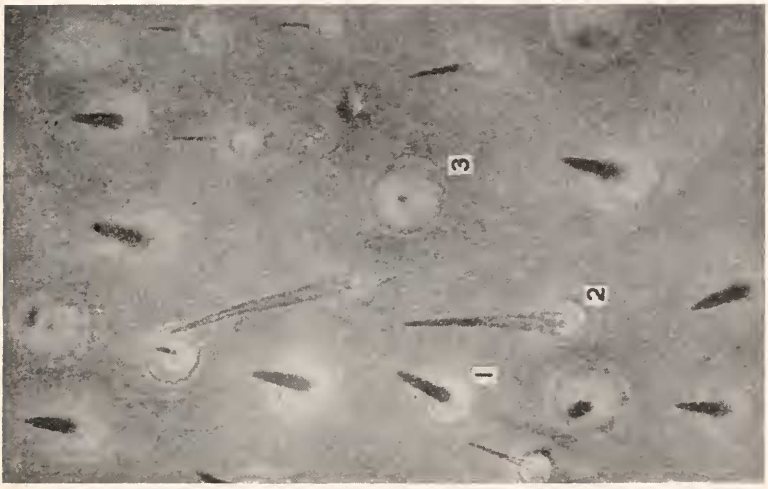

FIG. 1. Whole mount of small portion of surface of antennal flagellum of *Melanoplus differentialis* (Thomas) showing three types of chemoreceptors: 1, thin-walled basiconic pegs; 2, thick-walled basiconic pegs; 3, coeloconic pegs. Stained with crystal violet applied to *external* surface of intact antenna. × 560.

parts may be dissected if desired. This is best done at once before the material becomes hard and brittle. A grasshopper antenna, for example, may be cut into four or five pieces and each of these split lengthwise so that the cellular contents can be lifted out and discarded. When mounted external surface up in resin such pieces show the stained areas of the sense organs in sharp contrast against the clear, pale yellow, unstained cuticle (fig. 1). A series of such preparations stained for different periods of time will show not only the point at which the stain first penetrates but also its later progress from

that region. Each slide should be examined immediately since well-stained structures may lose their color within hours or days if any water remains in the tissue.

The method just described stains clearly all of the sense organs on the antennal flagellum of the grasshopper which earlier work had shown to be permeable in the living insect as well as those which other evidence obtained with the electron microscope (Slifer, Prestage and Beams, 1957, 1959) had indicated must also be permeable. A preliminary survey of antennae of species from all of the major insect orders has shown that they, too, possess sense organs which stain when crystal violet is applied to the external surface as described in this paper. Further work on these is in progress.

Summary

Whole insects, or their parts, which have been fixed in formalin can be stained with a solution of crystal violet applied to the *external* surface. Permanent preparations are made by drying small pieces of the body wall briefly in air, clearing in xylol and mounting in resin. The cuticle, itself, if uninjured, does not stain but dye enters at each of those points where any part of a tissue or cell is exposed. The technique is especially helpful in locating the distal tips of the neurones of chemoreceptors.

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